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The principal goal of this research was to determine if synthetic peptides derived from the helix-turn-helix domain of DNA binding proteins can be converted into a site specific nuclease by linking it to the nuclease activity of 1,10-phenanthroline-copper.

In this work, we have shown that a synthetic peptide derived from the helix-turn-helix binding domain of the *E. Coli* trp repressor can be converted into a chemical nuclease activity targeted for the aro H operator which is regulated by this control protein. The cutting by the peptide is not as specific as that observed by the chemically derivatized parent protein. Preferred scission is observed because DNA stabilizes the random coil protein in a helix-turn-helix conformation on its surface (eq.1a-c).

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peptide-OP (random coil) <----> peptide-OP(h-t-h)* {eq. 1a

*helix -turn-helix conformation

peptide-OP(h-t-h) + DNA operator sequence <----> {eq. 1b

peptide-OP (h-t-h)-- Operator sequence

Sequence specific scission would result from the following reaction:

peptide-OP (h-t-h)--Operator sequence--> {eq. 1c

nicked Operator sequence

Background scission is observed because of the high concentration of the peptide existing as a random coil

peptide-OP (random coil) + random sequence DNA--->

nicked DNA products {eq. 2

While our work suggests that 1,10-phenanthroline-modified peptides may not prove to be useful as reagent, it demonstrates that DNA binding can stabilize a 21 amino acid peptide in the helix-turn-helix conformation. It has also demonstrated that intact DNA binding proteins can be chemically be converted to specific stable nucleases.

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FINAL REPORT

SYNTHETIC SEQUENCE SPECIFIC NUCLEASES

ONR N00014-86-K-0524

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JULY 14,1989

The objectives of our initial proposal included:

1) Targeting DNA with native repressor proteins modified with 1,10-Phenanthroline-Copper.

2) Sequence Independent Scission using Peptides as Carriers of 1,10-Phenanthroline- Copper.

3) Synthesis of Sequence Specific Peptides modified with 1,10-Phenanthroline-Copper

1) *Targeting DNA with native repressor proteins modified with 1,10-Phenanthroline-Copper.*

Experiments with the *E. Coli trp* repressor demonstrated the feasibility of using a protein or a peptide as a carrier of the nuclease activity of 1,10-phenanthroline-copper. (C.-h. B. Chen and D.S. Sigman "Chemical Conversion of a DNA-Binding Protein into a Site-Specific Nuclease," Science 237 1197-1201 (1987)). The nuclease activity of the derivatized protein mirrored the binding specificity of the parent protein. Scission was observed at the two operators tested which are regulated by the native protein. In each case, L-tryptophan was required. This amino acid is a corepressor and must bind to the protein in order to achieve site-specific binding. An additional important feature of the cutting activity, essential for its projected use in chromosomal mapping, is that the reagent accomplished double strand scission.

Since one of the central goals of developing semisynthetic restriction endonuclease is to provide new reagents for chromosomal mapping, we are investigating the reactivity of the modified *trp* repressor within an agarose gel matrix. All manipulations of chromosomal size DNA must be carried out in the gel matrix to avoid shearing of the high molecular weight DNA. Presently, we are investigating the scission of the circular *E. coli* genome. Since there are three binding sites for the *trp* repressor in the *E. Coli* genome, it should be possible to identify three distinct segments of DNA by pulsed field gel techniques with appropriate probes in Southern blots.

To improve the efficiency of this scission reagent, 1,10-phenanthroline derivatives with long linker arms have been synthesized. They are currently being used to modify the *E. coli trp* repressor in order to improve the efficiency of the reaction.

2) *Sequence Independent Scission using Peptides as Carriers of 1,10-Phenanthroline- Copper.*

As initially proposed, we sought to examine the reactivity of 1,10-phenanthroline linked to a peptide which assumed a helical structure upon binding to DNA. This proposed line of experimentation led us to investigate the broader question of the influence of substitutents of 1,10-phenanthroline on the specificity of the nuclease activity. The following results have been obtained. 2-Substitution blocks scission since the coordination complex cannot undergo the oxidative cycle necessary for reaction. Substitutents at the 5-position are readily tolerated except if they are anionic. Substitution at the equivalent 4 and 7 positions with a methyl group yields an active complex; with a phenyl group, there is a change in reaction mechanism leading to fundamentally different nucleolytic activity. Substitution at the 3-position blocks scission. Substitution at the 5-position is not only tolerated but it also does not alter the intrinsic reactivity of the 1,10-phenanthroline-copper for a given DNA sequence if the substitutents lack defined specificity themselves. 5-Substitutents on 1,10-phenanthroline which have little influence on the specificity of the scission reaction include methyl, phenyl bromo, and acetamido, aminoethyl and amino hexyl groups. The reason for their lack of effect is apparent from the structure of the essential reactive intermediate formed between the tetrahedral 2:1 5-phenyl-1,10-phenanthroline-cuprous complex and DNA presented in Figure 1. In this model of the essential reactive intermediate, the 5-phenyl substituent does not interact directly with the walls of the minor groove. An important consequence of these studies is that the targeted nucleolytic agents should be synthesized by linking the affinity ligand to the 5-position of the phenanthroline.

Figure 1

The phenanthroline derivatives with 5-substituted cationic groups exhibited similar sequence preferences as the neutral derivatives but generally cut the less reactive sites more efficiently. A more even digestion pattern is consistent with minor groove binding of the coordination complex since the phosphodiester backbone generates a negative potential well. Therefore, cationic phenanthroline derivatives which form a cuprous complexes with net charges between +3 and +4 bind more efficiently than complexes formed with neutral phenanthrolines and (e.g. 5-phenyl-1,10-phenanthroline) with net charge of +1.

In order to examine the reaction of 1,10-phenanthroline-copper linked to a sequence neutral binding peptide, the coordination complex was attached to RecA, a bacterial protein which plays a central role in recombination and binds single and double stranded DNA in a sequence independent manner. Substitution of the phenanthroline at the 5 position with the RecA protein generated a very efficient scission reagent but one which lost the sequence specificity characteristic of the other 1,10-phenanthroline-copper complexes. The tight binding of the Rec A protein must override the small differences in binding affinity which lead to the sequence dependent reactivity. The experiments summarized in this section demonstrate that substitution at the 5-position of phenanthroline does not interfere with the nuclease activity. Moreover, these experiments demonstrated that the linkage of a small protein to the 1,10-phenanthroline-copper complex does not inhibit the nucleolytic activity either by scavenging cupric ion or quenching the oxidative intermediate essential for the DNA scission reaction.

3) *Synthesis of sequence specific peptides modified with 1,10-phenanthroline copper.*

In contrast to the results summarized above, derivatization of 1,10-phenanthroline at the 5-position with Hoechst dye 33258 yielded a 1,10-phenanthroline derivative with a scission specificity which reflected the A-T specificity of this fluorescent cytological stain (Figure 2). The cutting is particularly efficient because the dye binds within the minor groove.

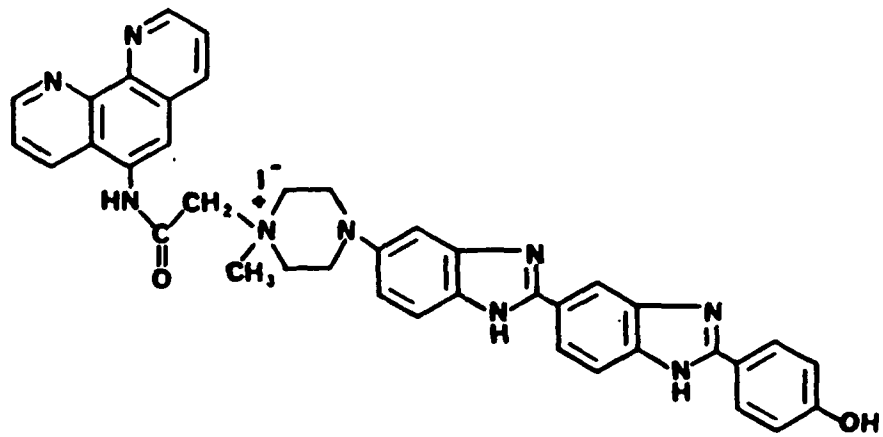

Figure 2

Hoechst dye 33258 is the first small organic ligand which we have studied that can redirect the cutting activity of 1,10-phenanthroline-copper

The experiments with Hoechst dye, as well as with the RecA protein, indicated a peptide derived from binding domain of a DNA binding protein should be able to target the chemical nuclease activity for site specific cleavage if these peptides had any affinity for DNA. Initially, it was proposed to synthesize peptides derived from the DNA binding domains of phage repressors. However, since we had demonstrated that the *trp* repressor could be transformed into a site specific nuclease, peptides derived from the DNA binding domain of this protein were synthesized instead. In this way, the relative efficiency of cutting of an operator sequence by the intact protein and the peptide could be compared. With the resources available, it was possible to synthesize the following peptides:

Wild type helix-turn-helix peptide

OP-Cys-Gln-Arg-Glu-Leu-Lys-Asn-Glu-Leu-Gly-Ala-Gly-Ile-Ala-Thr-Ile-

Thr-Arg-Gly-Ser-Asn -NH

Chemically mutated helix-turn-helix peptide

OP-Cys-Gln-Arg-Glu-Leu-Lys-Asn-Glu-Leu-Gly-Val-Gly-Ile-Ala-Thr-Ile-

Thr-Arg-Trp-Ser-Asn -NH

These peptide sequences correspond to the helix-turn-helix domain of the *trp* repressor with the following qualifications. The N-terminal cysteine residue is not part of the sequence of the protein but has been included to facilitate derivatization. The underlined residues at 77 and 85 in the wild type repressor are alanine and glycine, respectively. These have been substituted by valine and tryptophan, respectively for the following reasons: a) one mutant *trp* repressor with valine at 77 has a very high affinity for the operator sites; 2) the X-ray structure has shown that L-tryptophan, which must bind to the protein for the repressor to have affinity for DNA, interacts near this glycine and may be essential for stabilizing the conformation of the protein. The peptide derived from the wild-type sequence does not cause sequence dependent scission. Its scission pattern is that of unsubstituted 1,10-phenanthroline-copper.

Each peptide was a) dimerized by forming disulfide bonds at the N-terminal cysteine residue; and b) derivatized by 5-iodoacetyl-1,10-phenanthroline. The interaction of these various synthetic products with the *E. coli* aro *H* operator, one of three regulated by the *E. coli trp* repressor, was studied using DNase footprinting. Under conditions in which the native protein binds with high affinity, none of these peptides showed any sequence specific interaction with the target DNA using the DNase I footprinting assay (Fig 3).

The failure to observe high affinity binding does not necessarily preclude sequence specific cutting by the peptide. The free energy of binding of the peptide for its nucleotide sequence could stabilize the peptide in the conformation competent for DNA binding as indicated in the simple scheme below.

peptide-OP (random coil) <------> peptide-OP(h-t-h)* {eq. 1a

**helix -turn-helix conformation*

peptide-OP(h-t-h) + DNA operator sequence <--------> {eq.1b

peptide-OP (h-t-h)--Operator sequence

Sequence specific scission would result from the following reaction:

peptide-OP (h-t-h)--Operator sequence--> {eq.1c

nicked Operator sequence

A direct consequence of this simple scheme is that a peptide would bind to a specific sequence more weakly than a protein at comparable concentrations. Since site specific cutting is a reflection of binding, peptide directed cutting would not be expected to be as strong as that of protein directed cutting when presented at equivalent concentrations. The specificity of the cutting (i.e. the site of cutting) would depend on the reactivity of the 1,10-phenanthroline-modified random coil form of the protein free in solution.

In Figure 3, the scission of the nontemplate strand of the aro H operator using the OP-Cu derivatized trp repressor, underivatized OP-Cu, and OP-Cu derivatized chemically mutated peptide are compared. The products of the scission by the copper complex of OP-linked to the peptide were different from those of the unsubstituted 1,10-phenanthroline-copper exactly in the region in which the derivatized *trp* repressor cuts the nontemplate strand of the aro H operator most strongly (Fig 3). This was confirmed by comparing densitometric scans of the digestion pattern of OP-Cu, the peptide linked to OP-Cu and the *trp* repressor derivatized with OP-Cu (Fig.4). The targeted scission by the peptide indicated that the specific DNA sequence is able to stabilize the peptide in a precise conformation.

Peptide-Directed Scission of aro H

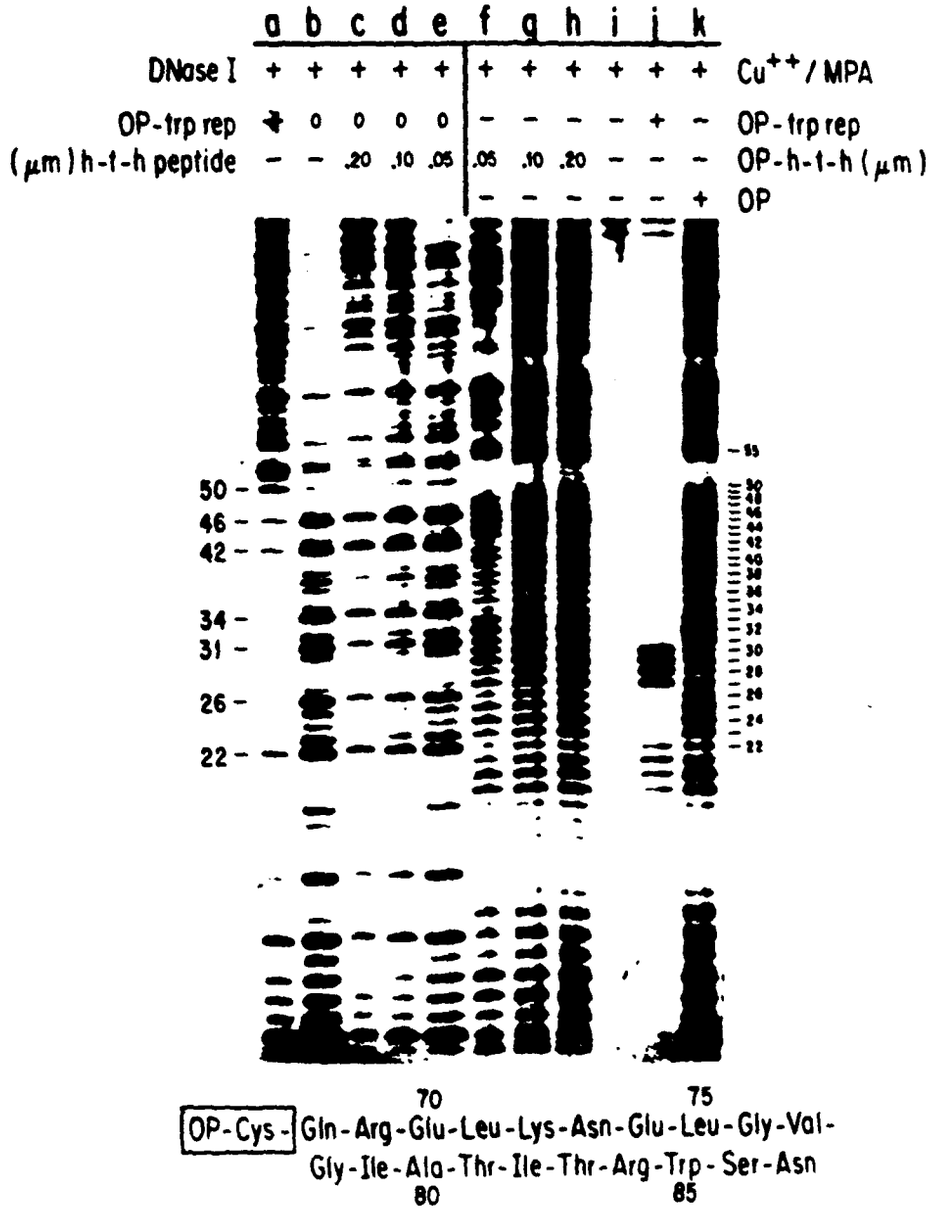

Figure 3

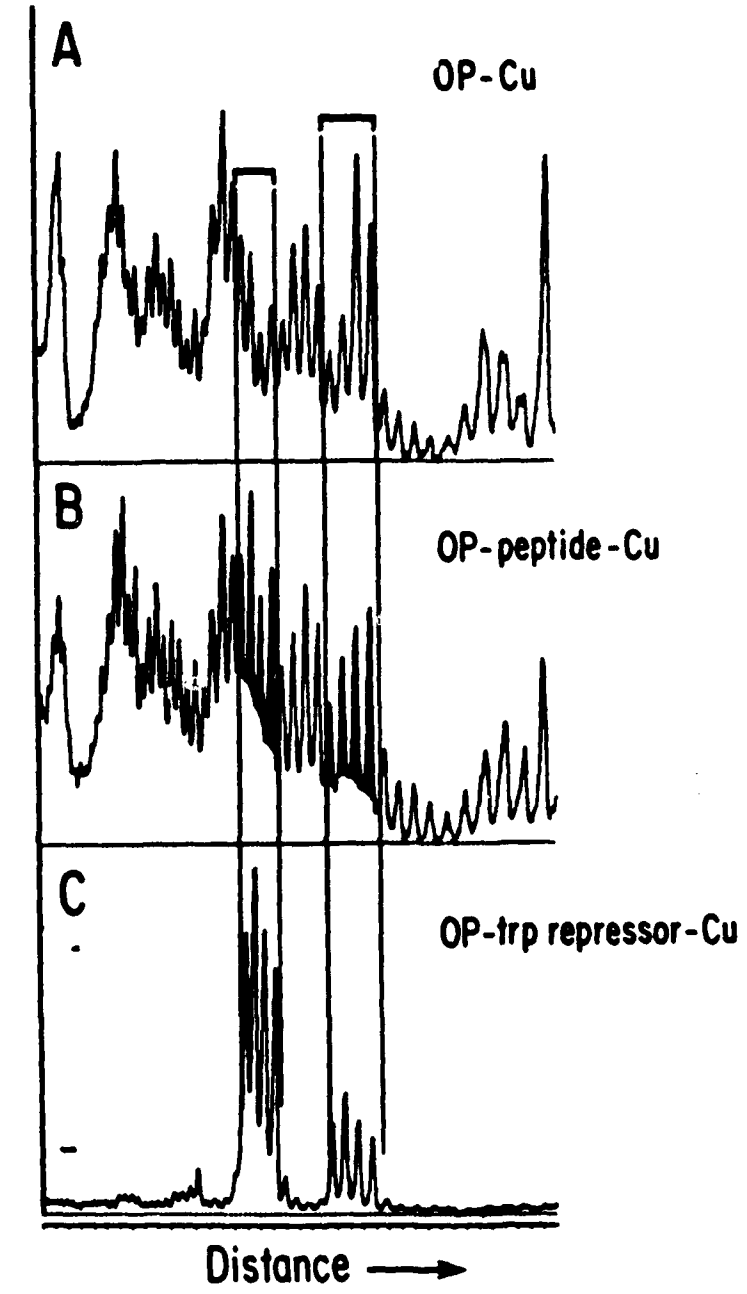
Peptide Directed Scission of aro H
A
OP-Cu
B
OP-peptide-Cu
C
OP-trp repressor-Cu
Distance

Figure 4

Even though enhanced cutting at the operator sites is evident, substantial background cutting is evident. This cutting is due to 1,10-phenanthroline linked to the peptide in the random coil conformation which like other 5-substituted phenanthrolines, reacts similarly to the unsubstituted 1,10-phenanthroline. Since the peptide is present at large excess relative to the target DNA, it is the predominant form of the peptide and 1,10-phenanthroline in the incubation mixture. As a result the large excess of peptide-OP (random coil) will result in the following reaction

peptide-OP (random coil) + random sequence DNA--->

nicked DNA products {eq. 2

The conformational instability of this peptide will limit the utility of peptides as sequence specific scission reagents. Binding energies between DNA and the peptide are not large enough to stabilize the conformation of the peptide at equivalent concentrations.***Specific cleavage will be achieved only if the peptide-OP (h-t-h) is intrinsically stable in the helix-turn-helix motif free in solution.*** Because of the intrinsic affinity of 1,10-phenanthroline-copper for DNA, it might not be possible to suppress the background reaction of the OP-Cu-peptide. Like the 21 unit described here, the 30 amino acid peptide derived from the DNA binding domain of the transcription factor TF IIIA also fails to exhibit site specific binding for its target sequence using a footprinting assay.

The signficant conclusion of our study is that it demonstrates that DNA binding can stabilize a 21 amino acid peptide in the helix-turn-helix conformation.